



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Hirokazu Inoue
Serial No.: 10/590,441 Art. Unit: 1636
Filed: August 23, 2006
Title: METHOD OF INDUCING HOMOLOGOUS RECOMBINATION
Examiner: Nancy Treptow Vogel
Docket No.: WING1-41066

DECLARATION

I, Hirokazu Inoue, hereby declare and state:

1. I am the inventor in the above-identified patent application Serial No. 10/590,441 entitled "METHOD OF INDUCING HOMOLOGOUS RECOMBINATION". I have reviewed the Office action dated March 3, 2009 and the references cited therein.

2. My educational background is as follows.

1974: The University of Tokyo, graduate school of science, Ph.D.
1968: The University of Tokyo, school of science, B.S.

My work experience is as follows.

2009 : "The Metzenberg Award" was conferred.
1995- : Professor at the school of Science, Saitama University
1980- 1995: Associate professor at the school of Science, Saitama University
1978- 1980: Assistant professor at the school of Science, Saitama University
1974- 1977: Researcher at National Institute of Environmental Health Sciences (NIEHS)

3. I conducted the following experiment to show that one of ordinary skill in the art reading the present specification could have identified and induced a decrease or loss of function of the genes *LIGIV* and *XRCC4* in filamentous fungi belonging to genus *Neurospora* or *Aspergillus* based on the known sequences of human counterparts to *LIGIV* and *XRCC4* and the known sequences of *Neurospora* and *Aspergillus* at the time the priority application was filed,

without using undue experimentation. Then one of ordinary skill could have introduced foreign DNA into the filamentous fungi, so as to conduct homologous recombination as claimed.

4. Certified Experiment Results

4-1. Increase in homologous recombination rate of *LigIV*-disrupted strain of *Aspergillus oryzae*

Preparation of *LigD* (*LigIV* homolog) gene-disrupted strain

A homolog (A0090120000322) of *Aspergillus oryzae* homologous to human *LIG4* was identified from the genome information of *Aspergillus*. This was designated as "*ligD*." A 1-kb portion on the 5'-terminal side of this gene and a 1-kb portion on the 3'-terminal side thereof were amplified by PCR, so as to produce a *ligD* gene-disrupting fragment having an sC marker as a selective marker. This fragment was introduced into the NS4 strain of *Aspergillus oryzae*. Such introduction was carried out by a protoplast method. A *ligD* gene-disrupted strain (Δ *ligD*) was identified from among a large number of transformants by PCR and Southern blotting.

Experiment of disrupting target *prtR* gene using *ligD*-disrupted strain as host

In order to measure the targeting efficiency of a wild-type strain and that of a Δ *ligD* strain, an experiment was carried out to target *prtR* as a regulatory gene of protease secreted outside the cell mass. With regard to DNA as a target, a pyrithiamin-resistance gene (*ptrA*) was used as a selective marker. DNA having homology to the *prtR* gene of a size of approximately 1 kb was constructed on the 5'- and 3'-terminal sides of the *ptrA* gene, and a transformation experiment was then carried out using this DNA. As a result, the targeting rate of the wild-type strain NS4 was found to be 8.3%, whereas that of the Δ *ligD* strain was found to be 96% (Table 1).

Table 1

Host strain	Number of transformants	Number of <i>prtR</i> -disrupted strains	Targeting rate (%)
NS4 strain	60	5	8.3
Δ <i>ligD</i> strain	55	53	96.4

4-2. Increase in homologous recombination rate of *Lig4*-disrupted strain of *Neurospora crassa*

Preparation of DNA construct for disruption of *Neurospora* *LIG4* homolog gene

A human *LIG4* homolog gene (hereinafter referred to as *ncLIG4*) was identified from the *Neurospora* genome database. A DNA fragment comprising an *ncLIG4* gene sequence was amplified by a PCR method using the genomic DNA of a wild-type strain of *Neurospora* as a template. The obtained DNA fragment was treated with HindIII, and it was then ligated to the HindIII site of pUC19. The obtained product was treated with EcoRV, and a hygromycin resistance gene was then inserted into a portion from which a 1.2 kbp *ncLIG4* gene had been eliminated. The obtained DNA was cleaved by treatment with HindIII, and the thus obtained DNA fragment was then introduced into a wild-type *Neurospora* strain by an electroporation method. A large number of transformed hygromycin resistance strains were extracted, and those whose *ncLIG4* gene had been substituted with a hygromycin resistance gene were

confirmed by PCR.

Preparation of *ncLIG4*-disrupted strain using bialaphos resistance gene

Using a bialaphos resistance gene, a *ncLIG4* gene-disrupted strain was prepared by a gene substitution method utilizing a homologous sequence. As shown in the figure below, such a *ncLIG4* gene-disrupted strain was prepared by substituting the hygromycin resistance gene of the construct as described above with a bialaphos resistance gene. Conditions for electroporation and operations to confirm gene substitution are the same as those in the case of using a hygromycin resistance gene.

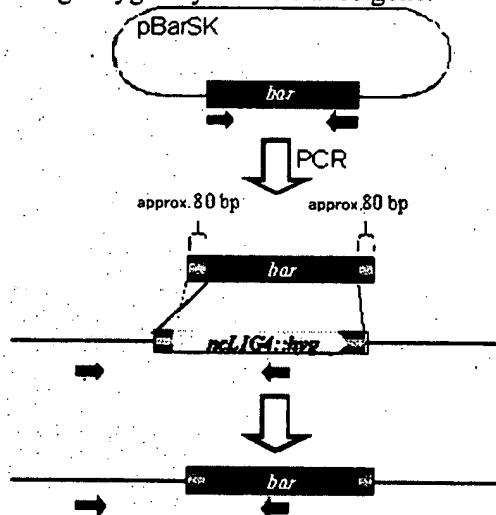


Figure: Preparation of *Neurospora ncLIG4* gene-disrupted strain using bialaphos resistance gene (*bar*) as marker

Experiment of disrupting *mtr* gene using *ncLIG4* gene-disrupted strain as host

An *os-2* gene and an *ad-3A* gene were selected as target genes. A hygromycin resistance gene was used as a marker, and a gene targeting construct was prepared such that it had *ad-3A* and *os-2* gene sequences on the 5'- and 3'-terminal sides thereof. The thus prepared construct was introduced into both a wild-type strain and a *ncLIG4::bar*-disrupted strain by the electroporation method.

An *os-2* gene-deficient strain is resistant to iprodione. Thus, a transformant, in which DNA insertion has taken place due to homologous recombination, is resistant to hygromycin and iprodione. An *ad-3A* gene-deficient strain has red-purple hyphae due to accumulation of intermediate products, and it has an adenine requirement. In an experiment using the two types of genes as target genes, the number of hygromycin resistance transformants in which homologous recombination insertion took place to the total number of hygromycin resistance transformants was calculated in terms of gene targeting efficiency (%) (Tables 2 and 3).

Table 2: Results of experiment targeting *os-2* gene

Host strain	Number of transformants	Number of <i>os-2</i> -disrupted strains	Targeting rate (%)
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Wild-type strain	67	10	15
Δ nclig4 strain	54	54	100

Table 3: Results of experiment targeting *ad-3A* gene

Host strain	Number of transformants	Number of <i>ad-3A</i> -disrupted strains	Targeting rate (%)
Wild-type strain	46	7	15
Δ nclig4 strain	38	38	100

4-3. Increase in homologous recombination rate of XRCC4-disrupted strain of *Neurospora crassa*

Gene targeting experiment using wild-type strain and *ncLIG1*-disrupted strain as hosts

Human *XRCC4* as a homolog gene of yeast *LIF1* (hereinafter referred to as *ncLIG1*) was identified from the *Neurospora crassa* genome database. A DNA fragment comprising an *ncLIG1* gene sequence was amplified by a PCR method using the genomic DNA of a wild-type strain of *Neurospora* as a template. The obtained DNA fragment was inserted into vector DNA.

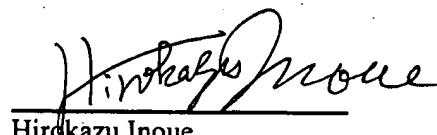
A hygromycin resistance gene was inserted into the *Xba*I site of the DNA, and DNA cleaved from the site with *EcoRV* was then introduced into a wild-type *Neurospora* strain by an electroporation method. A large number of transformed hygromycin resistance strains were extracted, and it was then confirmed by PCR that the *ncLIG1* gene had been substituted with a hygromycin resistance gene.

In addition, an *mtr* gene was amplified by PCR, and it was then subcloned into a vector. A bialaphos resistance gene used as a selective marker was inserted into a site 2 kb from the 5'-terminal side of the *mtr* gene and a site 2 kb from the 3'-terminal side thereof. The obtained DNA construct was introduced into a wild-type strain and an *ncLIG1*-disrupted strain. Thereafter, bialaphos resistance transformants were extracted, and the percentage of those whose *mtr* gene had been disrupted (those exhibiting resistance to p-fluorophenylalanine) was calculated (Table 5).

Table 5: Results of experiment targeting *mtr* gene

Host strain	Number of transformants	Number of <i>mtr</i> -disrupted strains	Targeting rate (%)
C1-T10-37 (WT) strain	85	15	17.6
Δ nclig1 strain	74	74	100

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


Hirokazu Inoue

Date: July 16, 2009